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Immunological Properties of Quillaja Saponins

Shahriar Behboudi

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Abstract

Quillaja saponin has for a long time been used as adjuvant in animal vaccines and is now a candidate for use in human vaccines. Quillaja saponin is extracted from the bark of the tree *Quillaja saponaria* Molina and consists of a glycosylated quillaic acid. Quillaja saponin is the unique component of the immunostimulating complex (iscom). Iscom is composed of Quillaja saponin, cholesterol, phosphatidylcholine and antigens. In general, Quillaja formulations i.e. free Quillaja saponin, iscom and iscom-matrix (antigen-free iscom) enhance both antibody and cell mediated responses but their capacities to enhance these responses differ. Iscom is the most potent of the Quillaja saponin formulations.

The proportion of the various Quillaja saponins in iscom-matrix or iscom has a profound effect on the immunoenhancing capacity of Quillaja saponin formulations. Therefore, it was crucial to design and employ methods to efficiently isolate and accurately quantify Quillaja saponin and lipids in iscom-matrix and iscoms.

A periodate oxidation procedure was employed to modify the monosaccharides of the carbohydrates of Quillaja saponin in a step wise manner. The modification resulted in decrease of adjuvant activity and toxic effects in a step wise manner, while cholesterol binding capacity remained unchanged.

Proinflammatory cytokines such as IL-1, IL-6 and IL-12 play important roles in the stimulation of B cell and T cell responses. These cytokines are produced by antigen presenting cells (APCs) upon stimulation with antigens and/or adjuvants. Comparative studies showed that iscoms were more efficient than other Quillaja saponin formulations in the induction of IL-1, IL-6 and IL-12 production. This result indicates that immunoenhancing capacity is greater when antigen and adjuvant are presented in the same particle. The capacity of iscom to stimulate IL-12 production is an important feature of its immunomodulatory activity.

QH-A was more efficient than QH-C in the induction of IL-1 and IL-6. This difference may explain the magnitudes of and/or the types of immune responses induced by these defined Quillaja saponins. Interestingly, the QH 7.0.3 formulation, when incorporated into iscom-matrix resulted in the most potent iscom-matrix formulation with respect to induction of APC to produce cytokines.

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Shahriar Behboudi
Department of Veterinary Microbiology
Uppsala

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Key words: APC, IL-1, IL-6, IL-12, iscom, iscom-matrix, periodate oxidation, Quillaja saponin

Author's address: Shahriar Behboudi, Department of Veterinary Microbiology, Virology Section, BMC Box 585, S-751 23 Uppsala Sweden.

To my parents

Contents

Appendix

Papers I-V

This thesis is based on the following publications and manuscripts which are referred to by their Roman numerals:

I, Behboudi S., Morein, B. and Rönnberg, B. Isolation and quantification of *Quillaja saponaria* Molina saponin and lipids in iscom-matrix and iscoms. Vaccine 1995, 17, 1690-1696.

II. Rönnberg, B, Makonnen, F., Behboudi, S., Kenne, L. and Morein, B. Effects of carbohydrate modification of *Quillaja saponaria* Molina QH-B fraction on adjuvant activity, cholesterol-binding capacity and toxicity. manuscript.

III. Behboudi, S., Morein, B. and Villacres-Eriksson, M. *In vitro* activation of antigen-presenting cells (APC) by defined composition of *Quillaja saponaria* Molina triterpenoids. Clin. Exp. Immunol. 1996, 105, 26-30.

IV Villacres-Eriksson, M., Behboudi, S., Morgan, A.J., Trinchieri, G. and Morein, B. Immunomodulation by *Quillaja saponaria* adjuvant formulations: *in vivo* stimulation of interleukin 12 and its effects on the antibody response. CYTKINE, 1997, 2, 73-82.

V. Behboudi, S., Morein, B. and Villacres-Eriksson, M. *In vivo* and *in vitro* induction of IL-6 by *Quillaja saponaria* Molina triterpenoid formulations. CYTOKINE, In press.

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Abbreviations

mAb	monoclonal antibody
APC	antigen presenting cells
BSA	bovine serum albumin
CD	cluster of differentiation
CFA	complete Freund's adjuvant
cpm	counts per minute
CSF	colony stimulating factor
CTL	cytotoxic T lymphocytes
DTH	delayed-type hypersensitivity
EBV	epstein-barr virus
ER	endoplasmic reticulum
ELISA	enzyme-linked immunosorbent assay
Fc region	fragment crystalline region of immunoglobulin
GM-CSF	granulocyte-macrophage-colony stimulating factor
gp	glycoprotein
HIV-1	human immunodeficiency virus-1
HPLC	high performance liquid chromatography
IFN-γ	interferon-γ
Ig-SC	immunoglobulin secreting cells
IL-	interleukin-
i.p.	intraperitoneal
i.v.	intravenous
LPS	lipopolysaccharide
MHC	major histocompatibility complex
NK	natural killer cells
OVA	ovalbumin
PBS	phosphate-buffered saline
PEC	peritoneal cells
s.c.	subcutaneous
TAPs	transporters associated with antigen presentation
TCR	T cell receptor
T_{H1}/T_{H2}	T helper lymphocyte type 1 or 2
TLC	thin layer chromatography
TNF	tumor necrosis factor
TD	T lymphocyte dependent antigen
TI	T lymphocyte independent antigen

Introduction

Background

One of the great achievements of human and veterinary medicine has been the development of the vaccine concept. Vaccines have traditionally been on the administration of whole live or dead micro-organisms, since the introduction by Jenner 200 years ago of a live vaccine against smallpox in man using a poxvirus naturally infecting another species i.e. cattle. Vaccines based on live replicating micro-organisms rendered non-pathogenic i.e. attenuated have been successful against a number of infectious diseases. Conventional attenuated live organisms have the potential to induce long-term immunological memory to and protection against pathogens. However, attenuated micro-organisms may, for several reasons cause infections, which may be because of insufficient attenuation of the pathogen, reversion of an attenuated pathogen to the pathogenic state or due to lack of an efficient immune system in immunosuppresed or immunodeficient individuals. Attenuated live vaccines may also be over-attenuated resulting in low immunogenicity. Killed whole micro-organisms such as viral particles or bacterial cells have been used in so-called inactivated vaccines to induce the protection against various infectious diseases such as foot-and-mouth disease in cattle and poliomyelitis in man. Thus, conventional vaccines have been effective against a number of infectious agents, mainly those causing acute diseases. However, even at this advanced state in vaccine development, we lack, in general, vaccines which induce protection against micro-organisms causing chronic or persistent infections. For that purpose a new generation of vaccines is required.

We consider the subunit vaccine approach a second generation vaccine because it is based on defined antigenic molecules and not whole micro-organisms i.e. specific antigens anticipated to induce protective immunity. Generation of non-protective and possibly detrimental immune responses directed against parts of the micro-organism which are not involved in the protective responses are thus avoided. Subunit vaccines are safe since they are not infectious. However, many antigens are poorly immunogenic because of their monomeric nature. In order to overcome this problem, the subunits need to be formulated into a multimer in particulate form. Furthermore, protective immune responses can be improved by the addition of adjuvants to the subunits in the construct in order to enhance their potency and to modulate the immune responses towards the type of response required for protective immunity. Modern techniques are now available to efficiently produce subunit antigens, plasmid DNA vaccines and live vector vaccines. However, with regard to the subunit vaccines, complementary techniques are required to convert the antigens to efficient vaccine immunogens. This thesis presents results of studies using the iscom as a delivery system for antigen and as an immunomodulator i.e. adjuvant.

Adjuvants

An adjuvant is defined as any agent or procedure which results in a specific increase in immunogenicity of vaccine components. Many adjuvants have been identified but none can be considered to be universally efficacious since each immunogen and targeted pathogen have their own requirements for the induction of protective immunity (Morein et al., 1996). It is most useful to classify adjuvants with regard to their functions. Three major mechanisms can be recognised by which adjuvants may exert their activities;

(a) physical presentation of antigen i.e. formulation of the antigen into small soluble particles, aggregates or organised multimeric structures, which includes the stabilisation and exposure of native conformational epitopes in the antigen.

(b) antigen targeting and antigen/adjuvant uptake, covering the depot effect, recruitment of APC, intracellular traffic, production of inflammatory cytokines from APC, increased antigen uptake and transportation to lymphatic tissues.

(c) immune potentiation/modulation, including intracellular traffic and processing of antigens, their association with MHC class I and II molecules and modulations of the T cell response towards Th1 or Th2 type of cytokines.

Many adjuvants function through more than a single mechanism and many have overlapping activities. The above listed areas of adjuvant activities do not exclude other functional aspects which may be of importance.

Quillaja saponaria Molina saponins

Quillaja saponin is extracted from the bark of a South American tree, *Quillaja saponaria* Molina (Dalsgaard, 1974). Although the adjuvant activity of saponin was recognised at the beginning of the nineteenth century with the discovery of experimental formol toxoid vaccines (Ramon, 1926), the saponin did not have any practical applicability until Espinet reported on its application in foot-and-mouth disease vaccines in 1951. Nevertheless, due to toxicity, its use has been limited to large animals e.g. in foot-and-mouth disease vaccines.
Quillaja saponin is a mixture of complex triterpenoid glycosides. The quillaic acid of the Quillaja saponin is glycosylated at triterpene carbon 3 and 28. The two structural features that distinguish Quillaja saponaria saponin from those of most other plant species are the fatty acid domain and the triterpene aldehyde at position 4 (Higushi et al., 1988).

Quillaja saponin is surface active compounds which binds to cholesterol, has the ability to cause hemolysis, and creates holes in biological membranes as revealed by electron microscopy (Campbell et al., 1992). Quil A is a semi-purified product obtained from extracts of crude Quillaja saponaria through dialysis, ion-exchange, and gel filtration chromatography. Quil-A and Spikoside are commercial names for semipurified Quillaja saponin (Quil A). The introduction of semipurified Quil A has brought consistency to the Quillaja based adjuvant formulation and reduced the local reactions seen after injection of crude saponin. Quil A has found widespread use as an adjuvant in animal vaccines (Dalsgaard et al., 1974). However, Quil A is by no means a homogenous compound and contains a number of related saponins. Use of organic solvents in reverse phase HPLC has facilitated the purification of several adjuvant-active saponins from Quillaja bark (Kensil et al., 1991; 1992; Rönnberg et al., 1995). These purified saponins named QS-7, QS-17, QS-18, QS-21 (Kensil et al., 1991), QH-A, QH-B and QH-C (Rönnberg et al., 1995) probably share a common glycosidic structure differing only in terminal monosaccharides (Higuchi et al., 1988). Each of these purified saponins was found to be adjuvant-active to a various extent, but differed with regard to other biological activities. For example, QS-18 and QH-B were found to be toxic for mice whereas QS-21, QS-7, QH-A and QH-C were less toxic (Kensil et al., 1991; Rönnberg et al., 1995). *In vitro*, free QH-A had low lytic activity compared to QH-C and QH-B.

To provide more efficient formulations of saponin, iscoms were constructed based on saponin, cholesterol and phospholipids with protein antigens incorporated in the same particle (Morein et al., 1984; Morein et al., 1996). Iscom particles without antigens incorporated are referred to as iscom-matrix. When QH-C and QH-B were incorporated into iscom-matrix the cytolytic activity was reduced (Rönnberg et al., 1995). This indicates that Quillaja saponin incorporated into iscoms or iscom-matrix can be used in human or animal vaccines with reduced local side effects seen in previous formulations.

Adjuvant activity

The B cell response

B cells are responsible for antibody production. They recognise antigens through antigen interaction with endogenously synthesised cell-surface immunoglobulin, which is their characteristic marker. B cells also express a variety of molecules such as MHC class II molecules, receptors for the Fc region of antibodies and complement receptors. The CD5 cell surface molecule,

which is expressed on most thymocytes and essentially all T cells, defines a B cell subset (called Ly-1 B cells in the mouse). B cells which do not express CD5 are called Ly-2 B cells and constitute the majority of the population. B cells commonly require both the presence of antigen and help from antigen-specific T cells in order to produce a specific antibody response. Antigens which cannot induce B cells to produce antibody without T-cell help are called T-dependent antigens (TD). However, there are antigens with the capacity to stimulate B cells directly which are called T-independent antigens (TI). The majority of protein antigens, however, are T-dependent. Many of the TI antigens, on the other hand, are large non-protein polymeric molecules with multiple copies of one or more antigenic determinants such as polysaccharides (Male et al., 1991; Mond et al., 1982).

Antibody responses to many TD antigens can be markedly enhanced by administration of the antigens together with other compounds known to have adjuvant activity. The addition of adjuvants to the antigens influences the antibody response with regard to magnitude, isotype distribution, avidity and sometimes the epitope specificity of the antibody responses (Gregoriadis et al., 1989; Keney et al., 1989; Van dam et al., 1989). The capacity of Quillaja saponin to enhance antibody responses has been demonstrated for a large number of antigens including both TD and TI antigens (Morein et al., 1995; White et al., 1991; Flebbe et al., 1986). Interestingly, different Quillaja saponin fractions i.e. QS-7, QS-17, QS-18, QS-21 (Kensil et al., 1991), QH-A, QH-B and QH-C (Rönnberg et al., 1995) give differing magnitudes of antibody responses when administered with TD antigens.

The TI antigens lack the capacity to induce memory responses, a fact which is due to these antigen`s failure to associate with MHC class II molecules. They do not exert the classical cognate T-B cell interaction which is essential for B cell Ig isotype switching and maturation into Ig-secreting cells (Male et al., 1991). The induction of antibodies to bacterial cell wall polysaccharides (Snapper 1996) and highly organised viral surface antigens (Mond, 82) such as soluble vesicular stomatitis glycoproteins can occur in the absence of T cell help. The T cell independent immunoglobulins are important in induction of protective immunity to extracellular bacteria (Snapper, 1996) and some cytolytic viruses (Bachmann et al., 1996). Generally, conventional adjuvants such as alum, do not enhance responses to TI antigens. In contrast, however, other adjuvants such as Quillaja saponin have been shown to enhance antibody responses of mice to TI antigens (Flebbe et al., 1986; deVelasco et al., 1994). Total antibody responses to polysaccharide were tenfold enhanced by the addition of Quillaja saponin after intradermal administration (Kensil et al., 1995). The antibody response to TI antigens enhanced by Quillaja saponin does not involve activation of T helper cells and, therefore, does not encompass an IgG memory response to the TI antigen. In general, it is suggested that Quillaja saponin can mediate part of its adjuvant effects primarily by a direct or indirect effect on B cells, stimulating production of cytokines acting in an autocrine or

paracrine fashion (Flebbe et al., 1986; Snapper, 1996). The possibility that the enhancing activity of Quillaja saponin on antibody responses may primarily be an indirect and due to effects of the adjuvant on natural killer cells (NK) and macrophages, should be considered. It has been shown that polysaccharide-specific B cells, activated through multivalent membrane bound immunoglobulin cross-linking by their corresponding antigen, may undergo some clonal expansion but will not secrete Ig unless one of a number of antigen-non-specific signals is also delivered. This second signal, which in itself, fails to induce significant polyclonal Ig secretion, may be delivered by NK cells or macrophages or through production of cytokines such as IFNγ and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Snapper, 1996). Polysaccharide-based experimental vaccines have been constructed by chemically coupling polysaccharides to protein carriers and incorporating these macromolecules into iscoms, as demonstrated with streptococcus pneumococcal type 14 capsular polysaccharide (Verheul et al., 1989). The objective of this is to induce T helper immune cells which on stimulation will potentiate B-cell responses to TI antigens.

In general, iscoms induce a classical transient IgM serum response followed by a high and long-lasting IgG response to TD antigens (Morein et al., 1996). More than a 10-fold enhancement of the antibody response is obtained compared with that induced by the same amount of antigen in a micelle form. Micelles have been used in comparative studies because the micelle is a particle similar in size to the iscom and presents the antigen as a multimer, this being an essential criterion for efficient presentation of an antigen in a vaccine. It has been shown that immunogenic properties of iscoms are affected by the ratio between antigen and adjuvant in the particles. The administration of iscoms with a high ratio of Quillaja saponin to antigen (Lövgren et al., 1996) or addition of Quillaja saponin in the form of iscom-matrix to iscoms with a low proportion of Quillaja saponin (Dotsika et al., 1997) induced substantially greater antibody responses than those seen after administration of iscoms with low ratio of Quillaja saponin to antigen. However, this effect was only marginal when high proportion of antigen was incorporated into iscoms (Lövgren et al., 1996).

High level antibody responses encompassing both specific IgG1 and IgG2a responses have been documented with various antigens incorporated into iscoms, and also antigens adjuvanted with iscom-matrix. This indicates the involvement of both Th1 and Th2 types of responses. In contrast, these same antigens administered without adjuvant stimulated an antibody response dominated by IgG1 (Morein et al., 1996). This influence of the adjuvant on the immune response is attributed to immunomodulation.

Studies on antibody responses to influenza virus iscoms administered s.c. measured at a single B-cell level in various lymphatic organs, including draining lymph nodes, spleen and bone marrow, show that antibody secreting cells persist for a long time and that clones of antigen-specific cells are strongly

expanded by a booster immunisation. After a single s.c. administration of flu-iscoms, the number of IgM secreting cells (IgM-SC) peaked at day 5 in both LN and spleen and declined rapidly thereafter. Specific IgG-SCs, on the other hand localised mainly in the draining lymph nodes, peaked at day 7 and then declined slowly.

A booster immunisation with flu-iscoms resulted in a rapid increase of the number of specific IgG-SC in both LN and spleen, with a peak response seen at day 4 at both lymphatic sites. Enhanced levels of antigen-specific IgG in serum were observed at day 4 and increased until day 8. The frequency of antigen specific IgG-SCs induced by flu-iscoms measured at day 7 was two times greater than that induced by flu-micelles in complete Freund's adjuvant (CFA) or TiterMaxTM and six-fold greater than that induced by flu-micelles in alum (Sjölander et al., 1996). It is of particular interest to note that after 50 days, 8 days after the s.c. booster immunisation, the majority of B cells with specificities for iscom borne antigen were found in the bone marrow while no homing to bone marrow was found for specific B cells induced by FCA (Sjölander et al., 1996).

Antigen presenting cells

Cells that can present antigen to lymphocytes in an immunogenic form are collectively termed antigen-presenting cells (APC). APC present antigen to T-cells which are either under MHC class I or MHC class II restriction (Male et al., 1991).

Endogenously generated antigen is processed in the cytosolic compartment of APC by degradation involving a multicatalytic proteinase complex, the proteasome. The resulting processed cytosolic peptides are transported into the endoplasmic reticulum (ER) lumen by ATP-dependent transporters associated with antigen presentation (TAP1 and TAP2). In the ER lumen, the peptides bind to nascent MHC class I molecules and are presented in association with MHC class I molecules for generation of $CD8^+$-mediated CTL responses.

Exogenously generated antigen enters the phagocytic pathway and is proteolyticly processed involving acid pH in cellular lysosomes. The processed peptides commonly consisting of approximately twenty amino acids are presented in association with MHC class II molecules for generation of $CD4^+$-mediated T helper cell responses which are pivotal in the induction and maintenance of antibody responses (Male et al., 1991).

However, recent experimental data have shown that in certain adjuvant formulations exogenous antigens can prime CTL response *in vivo*. For example antigens incorporated into iscoms induce CTL responses under MHC class I restriction (Takahashi et al., 1990). These observations suggest that iscom borne antigen reaches the cytosol of APC and is made available for processing by cytosolic protease and loading onto MHC class I molecules. To identify the

cellular compartments where iscom borne antigens are localised, and to compare this with the intracellular distribution of flu-micelles, Villacres-Eriksonn et al (1993) pulsed APC with biotinylated flu-iscoms or biotinylated flu-micelles and examined the distribution of antigen carried by the two different formulations. Two methods were employed to detect antigen: (a) streptavidin-gold conjugated particles were allowed to bind and were subsequently counted in the pulsed APC after visualisation by electron microscopy (EM) (b) the antigen-pulsed APC were lysed and the different cellular compartment were separated by differential centrifugation. Antigen was subsequently detected by a capture ELISA performed on the separated fractions. Both methods detected the antigen in the cytosol and in vesicles. Peritoneal cells pulsed with flu-iscoms showed a 50-fold greater concentration of scattered gold particles distributed in clear and dense vesicles and also distributed in the cytosol when compared with flu-micelles (Villacres-Eriksson, thesis 1993). The EM results suggest that iscoms can integrate into plasma membranes as well as into the phagosomal membranes and thereby expose antigens to the proteolytic enzymes in the cytosol, facilitating MHC class I presentation. These results also demonstrate the high capacity of iscoms to be taken up by macrophages. Efficient uptake and internalisation of iscom borne antigen has also been demonstrated for dendritic cells and B cells, suggesting that selective uptake by APC is an important factor in enhancing the immunogenicity of iscom borne antigens (Villacres-Eriksson, thesis 1993).

An alternative route for the priming of MHC-I-restricted CTLs by exogenous antigen was recently revealed when the existence of a phagosome-to-cytosol pathway was discovered. Apparently, some antigens can escape from phagosomes into the cytosolic compartment, where they presumably enter the endogenous processing pathway (Jondal et al., 1996). The question of whether this mechanism is employed by iscoms deserves more study.

The upregulation of MHC class II expression on APC is probably an important contributing factor in the adjuvant activity of Quillaja saponin formulations. This has been shown with soluble Quillaja saponin (Heath et al., 1991) and iscoms (Watson et al., 1992; Bergström et al., 1992) and is IFNγ dependent (Bergström et al., 1992). Quite unexpectedly van Binnendijk et al., demonstrated that the presentation of iscom borne antigen (Measles virus iscoms) by MHC class II molecules is not perturbed by chloroquine, which blocks protein degradation in lysosomes by raising lysosomal pH. This finding suggests that iscom borne antigens can target both endosomal and non-endosomal pathways for presentation of peptides by class II-molecules.

Antigen presenting cells (APC) pulsed with iscoms carrying influenza virus proteins are able to stimulate unprimed splenocytes to proliferate. The proliferative response was considered to be a primary antigen-dependent stimulation, since APC pulsed with iscom-matrix not containing antigen did not induce proliferation of unprimed splenocytes (Villacres-Eriksson, thesis 1993).

Since neutralising antibodies to IL-1, IL-6, GM-CSF and TNFα significantly inhibited antigen driven primary T cell proliferation when added to the culture, this priming was considered to be dependent on the production of these proinflammatory cytokines by macrophages stimulated with iscoms. The capacity of protein antigens supplemented with iscom-matrix or with free Quillaja saponin to stimulate APC to prime splenocytes to proliferate has not yet been sufficiently tested.

Several factors of importance for the initiation of immune responses such as proinflammatory cytokines are released from APC. Gebara et al. (1995) could detect TNF activity in sera from mice administered s.c. with free Quillaja saponin or BSA. The level of TNF activity in sera of mice administrated both with BSA and saponin was equal to that seen after administration of saponin alone. Interestingly, the capacity of saponin to induce TNF activity, *in vivo*, was completely abolished in mice if saponin was injected together with *Crotalus durissus* venom demonstrating that this antigen strongly interfered with the effects of saponin on the response of APC. Furthermore, the capacity of iscoms to stimulate APC to release cytokines, such as IL-1, IL-6 and GM-CSF has been previously reported. But, TNFα activity was, however, only detected indirectly when it was seen that anti-TNFα antibody inhibited the proliferation of primed splenocytes after restimulation *in vitro* (Villacres-Eriksson et al., 1993; Villacres-Eriksson, thesis, 1993).

The T cell response

T cells are lymphocytes which develop and differentiate in the thymus before seeding the secondary lymphoid tissues. They recognise processed peptides associated with MHC class I or class II molecules via a specific cell-surface molecule, the T cell receptor (TCR). This receptor consists of an antigen-binding portion formed by two different polymorphic chains. The antigen binding portion of the TCR complex either consists of an αβ heterodimer or a γδ heterodimer. The great majority of peripheral T cells have αβ chains and most of them are either $CD4^+$ T helper cells or $CD8^+$ cytotoxic cells. $CD8^+$ T cells recognise antigen associated with MHC class I molecules, while the $CD4^+$ T cell interacts with APC presenting the processed antigen associated with MHC class II molecules (Male et al., 1991).

In mice, T lymphocytes are classified into two groups, depending on the cytokines they produce, i.e. Th1 or Th2 type cytokines. Interleukin 2 (IL-2), interferonγ (IFNγ) and tumour necrosis factor β (TNFβ), are classified as Th1 type cytokines while IL-4, IL-5 and IL-10 are classified as Th2 type cytokines. Th1 type cytokines are involved in cell-mediated inflammatory reactions such as the activation of cytotoxic and inflammatory functions and induction of delayed-type hypersensitivity (DTH) reactions.
Th1 type cytokines, e.g. IFNγ provide help for B cells and stimulate production of antibodies of a specific subclass in mice IgG2a. However, high concentration of Th1 type cytokine producing cells, can induce suppression. Th2 type

cytokines are commonly found in association with strong antibody, specifically IgE, responses (Mosmann et al 1995).

The kinetics of clearance from the site of administration after subcutaneous administrations (s.c.) and the subsequent organ distribution of radioactive influenza virus antigen incorporated into iscoms or administered with complete Freund's adjuvant (CFA) (Lövgren et al., 1996; Sjölander et al., 1997) clearly indicate that the iscoms efficiently target the antigen to the lymphatic system. Following s.c. administration of iscoms containing radioactively labelled influenza antigens, strong radioactive signals were observed in blood, liver and draining lymph nodes of mice. Much weaker signals were detected in blood or other organs when radioactive flu-micelles in CFA were injected. This finding is well in line with the previous findings that antigens administered in oil emulsions are retained at the site of injection, the so-called depot effect. Also, i.p. administered flu-iscoms more efficiently localised the radiolabelled antigen to the draining lymphatic organ i.e. spleen, where it remained for a longer period of time than when administered in the form of flu-micelles (Watson et al., 1989).

Sjölander et al. also studied the kinetics and organ distribution of T cell responses in the draining lymph nodes and spleen after a single s.c. injection of flu-iscoms and compared this to the response after injection of the same antigen in the micelle form supplemented with FCA. The T cell response as measured by proliferation of T cells and cytokine production was first seen in draining lymph nodes. This localisation of T cells was transient, peaking 5 days after administration. Five days later, i.e. 10 days after administration, the T cell response peaked in the spleen where the level remained stable for a longer period of time while vanishing in the LN. Corresponding analyses of T cell responses in spleen and draining LN of mice administered s.c. with flu-micelles in FCA revealed that the antigen specific T cell response mainly accumulates and persists in the draining LN. The above findings indicate that the choice of adjuvant and the composition of adjuvant formulations influences not only the magnitude of T cell responses but also the localisation and type of T cell responses i.e. Th1 or Th2 type of response.

Several articles have been published concerning the effect of Quillaja saponin on T cell responses (Fossum et al., 1990; Villacres-Eriksson et al., 1992; 1995; Maloy et al., 1995; Lövgren et al., 1996; Sjölander et al., 1997; Kensil et al., 1995). In general, Quillaja saponin incorporated into iscoms carrying antigens in the same particle stimulates both T helper cells and cytotoxic T cells under MHC class II and MHC class I restriction respectively (Morein et al., 1995). It has been well documented that iscoms induce a higher T-cell response than antigen without adjuvant as measured by a proliferative assay following restimulation of splenocytes with specific antigen *in vitro* (Villacres-Eriksson et al., 1992; 1995; Maloy et al., 1995; Sjölander et al., 1997 in press). It is also reported that iscoms induce both Th1 and Th2 types of responses as measured

by the antigen driven production of IL-2, IFNγ, IL-5 and IL-4 assayed in the supernatants of primed T cell cultures (Morein et al., 1996; Maloy et al., 1995). Interestingly, higher amounts of IL-2 and IFNγ were measured in the supernatant of splenocytes from iscom-primed mice which were restimulated *in vitro* with flu-iscoms than was measured in cells from mice restimulated with flu-micelles.

With regard to IL-10 production, flu-micelles were more efficient than flu-iscoms in priming T helper memory cells. On the other hand, the IL-4 level in the supernatant of *in vitro* restimulated splenocytes from mice primed with iscoms was not different from that measured in cells from mice primed with flu-micelles (Villacres-Eriksson, 1995).

Splenocytes from mice immunised with different antigens i.e. *Trypanosoma cruzi* iscoms (Carlomagno et al., manuscript), EBVgp340 iscoms (Dotsika et al., 1997) or flu-iscoms (Villacres-Eriksson, 1995) showed different patterns of cytokine production after *in vitro* restimulation. With flu-iscoms, IL-2, IL-4 and IFNγ were detected in culture supernatant. With *Trypanosoma cruzi* iscoms, IL-4 and IFNγ were not detectable in the culture after restimulation. However, when an immunosuppresive antigen from the flagellar fraction of *Trypanosoma cruzi* was depleted and the remaining antigens administered with iscom-matrix, a high production of INFγ was detected after *in vitro* restimulation of splenocytes. With EBVgp340 iscoms, IL-2 and IFNγ were induced but not IL-4. Interferonγ was produced by restimulated splenocytes only when an immunosuppressive antigen was depleted and administered with iscom-matrix (Hansen et al., 1996). The above results demonstrate that the intrinsic biological activities of protein antigens have a clear influence on the immunomodulatory properties of Quillaja saponin.

As discussed above, the presence of antigen and adjuvant in the same particle increases the magnitude of B cell responses. The same is true for T cell responses. The magnitude of the T cell response, as measured by cytokine production, in mice immunised with flu-iscoms was considerably higher compared with that seen after immunisation with iscom-matrix and antigen in separate entities (Lövgren et al., 1996). Greater doses of Quillaja saponin were required to be incorporated into iscom-matrix in order to induce the same levels of T cell responses as those induced by iscoms. These results again demonstrate enhanced adjuvant activity of Quillaja saponin seen when adjuvant and antigen are in the same particle. In general, however, the type of response was similar.

In vivo, high levels of Th1-type cytokines plus moderate amounts of Th2 type cytokines were detected in the sera of mice administered s.c. or i.m. with influenza virus antigens supplemented with QH-C (In this article the QH-C was called LT-C). In contrast, other adjuvants used in this study i.e. MF59, MF59 containing MDP, MF59 plus lipid A and alum, induced only Th2 type cytokines when administered with influenza antigens (Valensi et al., 1994). The ability of

Quillaja saponin to promote the Th1 type of response was shown in an antigen specific manner, IFNγ being detected in the sera of mice after three s.c. administrations of bovine serum albumin supplemented with Quillaja saponin. However, the administration of *Crotalus durissus* venom as antigen either alone or with saponin, was unable to stimulate IFNγ production as was previously described for TNFα production, again suggesting that the immunomodulatory effects of saponin are influenced by the accompanying protein antigens (Gebara et al., 1995). It should also be mentioned that various fractions of Quillaja saponins i.e. QS-7, QS-17, QS-18, QS-21, QH-A, QH-B and QH-C may have different immunomodulatory effects. These effects will be discussed later in the text.

The immunomodulatory effects of Quillaja saponin or IFNγ on lymphocyte traffic, MHC class II expression on adherent peritoneal cells and their respective adjuvant activities have been studied in different strains of mice. In one study Heath et al. (1991) showed that intradermal injection of saponin or IFNγ increased homing of radiolabelled T cells to the injection sites. Likewise after i.p. injection there was an increased MHC class II expression on adherent peritoneal cells. The strong homing effect seen on lymphocytes was most prominent in those strains of mice which showed a low response to the adjuvant and these mice did not develop protective immunity against *plasmodium yoelii.* These results indicate that a prominent homing had an inverse adjuvant effect. The adjuvant activity of Quillaja saponin generally mimicked that of IFNγ from the point of view that both induced similar patterns of immune response as measured by protection, T cell homing and expression of MHC class II on PEC in various strains of mice. These results indicate that Quillaja saponin may act, at least partially, by the induction of IFNγ production (Heath et al., 1991). However, this hypothesis does not correspond with the result reviewed above showing that saponin administered alone without antigen did not induce detectable IFNγ in serum samples. The possibility should however be considered that the level of locally produced IFNγ is not sufficiently high to be detected in serum.

Antigen-specific cell-mediated immune responses are considered to be crucial for protection against various viruses and intracellular parasites (Male et al., 1991). The contribution of MHC class I restricted $CD8^+$ cytotoxic T lymphocytes (CTL) to protection, and limitation on the induction of these effectors are topics which deserve much consideration in the design of vaccines. The first report of induction of virus-specific CTL responses by an exogenous antigen was that of Deres et al. (1989). An efficient influenza virus specific CTL response was induced in mice by a synthetic lipopeptide experimental vaccine.

The capacity of Quillaja saponin, peptides encapsulated in Quil A-containing lipisomes (Lipford et al., 1994; 1994) or iscoms to induce MHC class I restricted CTL has been demonstrated with various antigens (Jones et al., 1988;

Hsu et al., 1996; Takahashi et al., 1990; Mowat et al., 1991; 1993; Trudel et al., 1992; Van Binnendijk et al., 1992). Takahashi et al. (1990) presented the first inclusive evidence that iscoms induce CTL responses, demonstrating that CTL could be induced by iscoms containing HIV gp 160 or influenza virus envelope proteins. Later, CTLs have been seen to be induced by a number of other antigens incorporated into iscoms, e.g., influenza virus glycoproteins (For review see Morein et al., 1995), measles virus F-protein (Van Binnendijk et al., 1992), heat shock protein of mycobacterium (Zügel et al., 1995) and ovalbumin (Mowat et al., 1991; Heeg et al., 1991). The very first report which indicate that CTL could be induced by iscoms was however that published by Jones et al. (1988) who used an intranasal administration system to demonstrate this. The capacity of iscoms to induce CTL via the mucosal route was later confirmed by Mowat et al. (1991) using OVA-iscoms administered orally. The *in vitro* stimulation of naive $CD8^+$ T cells with a $CD4^+$ population of lymphocytes taken from iscom primed mice did not result in generation of specific $CD8^+$ CTL. However, the combination of primed $CD4^+$ cells with primed $CD8^+$ cells induced the highest killing rate seen in the study presumably because of the production of some cytokines, by $CD4^+$ cells e.g. IFNγ, which enhanced generation of cytotoxic T cells.

A great many experimental vaccine formulations have been reported to induce CTL. In general, very high doses and repeated administrations were involved in these studies. Such results indicate that any antigen formulation might induce CTL when animals are overloaded with high doses of antigen and administered repeatedly. With this in mind, CTL responses have been reported against ovalbumin (Newman et al., 1992), gp 160 of HIV-1 (Wu et al., 1992) and p55 gag or gp120 env proteins from the mac251 strain of the simian immunodeficiency virus (Newman et al., 94). In these experiments protein antigens and free Quillaja saponin QS-21 were co-administered in mice. To obtain CTL, however, three immunisations at two week intervals with 25 µg/dose of OVA adsorbed to 250 µg/dose of Al(OH)3-gel plus 20 µg/dose of QS-21 were required (Wu et al., 1994). In contrast, antigens incorporated into iscoms induced high levels of MHC class I restricted CTL after just one immunisation at a low dose of antigen (Takahashi et al., 1990; Mowat et al., 1991; Heeg et al., 1991; Maloy et al., 1994). The administration of 0.1 µg/dose of flu-iscoms has been shown to induce potent and specific CTL responses in mice after just one immunisation (John Cox, personal communication). These findings demonstrate that more efficient induction of CTL responses can be achieved when antigen and adjuvant are in the same particle.

Aims of the present study

The main objective of this thesis work has been to further clarify the immunological properties of Quillaja. saponins. More specifically my aims have been:

1. To design methods to quantify the various components incorporated into iscom-matrix and iscoms.

2. To analyse the effects of chemical modification of monosaccharides of Quillaja saponin on its biological activities.

3. To analyse the activation of antigen presenting cells by defined Quillaja saponins.

Results and Discussion

Isolation and quantification of saponin and lipids in iscom-matrix and iscoms (paper I)

In the iscom, multiple copies of antigen are attached by hydrophobic interactions to a matrix composed of Quillaja saponin and cholesterol which is an obligatory component and another non-obligatory lipid e.g. phosphatidylcholine. A great number of articles have been published about iscoms but information on and methods for the quantification of components incorporated into iscoms were lacking. The amount and the ratio of each saponins as well as the amount of antigens incorporated into iscoms are important factors which influence immunogenicity, the immunomodulatory properties and toxicity of the formulations (Lövgren et al., 1996). In addition, the source of lipids and type of lipids in iscoms or iscom-matrix may also have effects on their adjuvant activities (Åkerblom and Behboudi, unpublished results). Therefore, it is essential to quantify the amount of each component in iscoms and iscom-matrix.

Isolation of iscom-matrix and iscom components

To be able to quantify lipids, Quillaja saponin and proteins methods have to be employed to extract and isolate these various substances. A chloroform-methanol-water extraction procedure initially described by Wessel and Flügge (1984) for the isolation and recovery of protein was employed. This method enables proteins to be isolated from dilute solutions, for example column chromatography effluents in which the protein is mixed with lipids and detergents. We followed this extraction procedure using chloroform-methanol-water in the proportions one: four: one in order to isolate the iscom components. Saponins were totally isolated in the methanol phase (i.e. triterpenoid phase) and lipids in the chloroform phase. After further addition of methanol to the chloroform phase (1:1) a proportion of the protein was precipitated and all lipids were recovered in the supernatant, i.e. the lipid phase. Proteins were, however, detected in all phases i.e. the triterpenoid phase, the lipid phase and in the precipitated protein. In conclusion, a chloroform-methanol-water method can be used to efficiently isolate saponin and lipids incorporated into iscoms but this method is not suitable for isolation of proteins incorporated into iscoms.

Quantification of Quillaja saponin in the triterpenoid phase

Quantification of saponin was carried out using reverse phase HPLC (column; LiCrospher 100 RP-18) or by a calorimetric method utilising orcinol sulfuric acid. Since the calorimetric method measures carbohydrates, the orcinol sulfuric acid also identifies carbohydrates from other sources e.g. glycoproteins such as gp120 from HIV-1 or any other glycoprotein, which is incorporated into iscoms. Using HPLC, the recovery of saponin in iscom-matrix measured as a percentage of the starting material ranged from 82 to 105%, and in the iscoms

the recovery ranged from 60 to 100%. A simple method to measure the total quantity of saponin, if quantification of particular Quillaja saponin is not required, is to measure the absorbance of the triterpenoid phase at 206 nm in a spectrophotometer. For isolation and quantification of each of the Quillaja saponin separately HPLC is the method of choice. The QH-A and QH-C fractions in the triterpenoid phase were separated by reverse phase HPLC (column; LiCrospher 100 RP-8) and the components were identified by thin layer chromatography (TLC) using the free form of the respective components as standard. Since QH-C is more hydrophobic than QH-A, it was anticipated to incorporate into iscom-matrix or iscoms more efficiently than QH-A. This was not the case with flu-iscoms. The ratio of QH-A to QH-C in the flu-iscoms was similar to that in the starting material. However, other factors than the Quillaja saponin e.g. chemical properties of lipids and/or antigens in iscoms, may also influence the ratio of each Quillaja saponin recovered from iscoms.

Quantification of lipids in the Lipid phase

Cholesterol and phosphatidylcholine in the lipid phase were isolated and quantified by HPLC (column; Lichospher Si-60). The complete separation of cholesterol and phosphatidylcholine was traced and confirmed by TLC and by the use of cpm values trace amounts of radioactivity added to each lipid component prior to incorporation i.e. either of 3[H] cholesterol or 3[H] phosphatidylcholine. The cholesterol content was measured according to its peak height. The phosphatidylcholine peak was measured by the cut-and-weigh method i.e. by cutting out the area under the peak diagram and weighing it. The recovery of lipids calculated from the starting concentration in the iscom-matrix samples was about 70-76% for cholesterol and 21-26% for phosphatidylcholine using the HPLC assay. Thus, the recovery of cholesterol incorporated into iscom-matrix or iscom preparations was approximately two- to three fold higher than that of phosphatidylcholine. The efficiency of incorporation of cholesterol can be explained by the strong and specific binding capacity of this lipid to saponin (For review see Höglund et al., 1989) and by the possibility that there was no surplus of cholesterol in the starting material for the iscom-matrix preparation. Phospholipids are incorporated by hydrophobic interactions.
The cholesterol content in the lipid phase was also determined using a calorimetric assay based on oxidation of cholesterol by cholesterol oxidise. In the presence of catalyse, the hydrogen peroxide produced in this reaction oxidises methanol to formaldehyde. The latter reacts with acetylacetone creating yellow colour.

Comparison of three different methods for the quantification of lipids

The radioactivity of labelled lipids shows the exact amount in iscom-matrix or iscoms. Therefore, we used the cpm value of labelled lipids to confirm the accuracy of HPLC and the calorimetric method for the quantification of lipids. The percentage of cholesterol recovered from iscom-matrix was 70 to 76% measured by HPLC, 55 to 58% by calorimetric assay and 58 to 72% by measurement of radioactive incorporation. The percentage of

phosphatidylcholine recovered from HIV-1 iscoms was 17% measured by both HPLC and radioactivity, verifying the accuracy and suitability of HPLC as a standard method. Since, the radioactive signals generated by 3[H] cholesterol and 3[H] phosphatidylcholine can not be differentiated in a radioactive assay, quantification of lipids by HPLC is the most practical and accurate method.

Quantification of proteins in iscoms

Two methods were used to measure protein content i.e. the Lowry method and amino acid analysis. In the Lowry assay, protein which is to be assayed is treated with copper in alkali and is subsequently used for reduction of phosphomolybdic-phosphotungstic reagent, giving yellow colour which can be quantified. The amino acid analysis quantifies each amino acid by HPLC. There was a discrepancy between the Lowry method and amino acid analysis in measuring protein content. The Lowry method consistently gave a higher value than the amino acid analysis. We conclude that the Lowry method is not suitable for quantification of proteins from iscoms isolated by the Wessel and Flügge precipitation procedure. The amino acid analysis is the method of choice for quantification of proteins both from iscoms isolated by the procedure described above and for non-separated iscom preparations. Furthermore, the chloroform-methanol-water extraction procedure used for isolation of different iscom components is not suitable for the isolation of proteins. Proteins were distributed in the all phases i.e. the triterpenoid phase and lipid phase as well as in the protein precipitate.

Modification of the carbohydrate moiety of saponin influences their adjuvant activity and toxicity but not their cholesterol-binding capacity (paper II)

The unique constituents of the iscom are Quillaja saponin which consist of a quillaic acid, a fatty acid domain and two branched carbohydrate chains of monosaccharides (Higuchi et al., 1988). The latter have been considered to be crucial components involved in the adjuvant activity and in the toxic effects of Quillaja saponin. Therefore, modifications of the various monosaccharides of Quillaja saponin might shed light on the effects of carbohydrates on the biological activity of Quillaja saponin. QH-B is a defined Quillaja saponin fraction with high adjuvant activity showing toxicity in mice at doses ranging from 50 to 200 μg per dose (Rönnberg et al., 1995). QH-B was treated with sodium periodate at varying concentrations ranging from 2.5 to 50 mM at acidic pH (pH=4.5). The effects of periodate treatment on the sugar residues, molecular weight, toxicity, adjuvant activity and cholesterol binding capacity of modified QH-B were analysed.

Treatment of sugar with periodate cleaves the carbon-carbon links between vicinal hydroxyl groups with the subsequent formation of two aldehyde groups. The latter can be involved in hemiacetal formation with adjacent hydroxyl

groups and thus can form new rings differing from the original sugar (Bobbitt, 1956).
The sugar analyses of periodate-modified QH-B were performed by a chemical treatment of the sugars which converts only the modified sugars to their alditol acetates. Briefly, the samples were hydrolysed with aqueous 2M trifluoroacetate acid and the released sugars converted to their alditol acetates by reduction with sodium borohydride and subsequent acetylation. The alditol acetates were analysed by gas liquid chromatography-mass spectrophotometry and the amounts of each sugar estimated relative to the internal standard sample (inositol). The results showed that focuse and arabinose were only marginally affected, approximately 60%, of the rhamnose and glucuronic acid was affected to whereas most of the xylose, galactose and apiose was destroyed. The latter were totally destroyed even at the lowest concentration (2.5 mM) of sodium periodate. Xylose, galactose and apiose have all been shown to be terminal groups in the structure of a similar saponin obtained from Quillaja saponaria Molina (Higuchi et al., 1988). The terminal positions may well explain that the greatest modification effects were seen in these three sugars. From the above results, we can postulate that any differences in the biological activities of modified QH-B may be at least partially due to the modification of these three monosaccharides.

The molecular weight of the modified QH-B was also analysed by mass spectrophotometry. There was no change in the molecular weight after treatment of QH-B with periodate since the peaks in the spectra remained at the same masses. Therefore, it was concluded that no loss of sugar residues had occurred. However, the peaks seen on analysis of treated QH-B with 50 mM sodium periodate were broader than non-modified QH-B, demonstrating an even more complex mixture of modified saponin.

The adjuvant activity of modified QH-B was determined by measuring total antibody responses in mice immunised s.c. with flu-micelles adjuvanted with non-modified or periodate modified QH-B. The highest antibody levels were found in mice when antigen was administered with non-modified QH-B and the lowest levels were seen with antigen co-administered with QH-B modified with 25 or 50 mM sodium periodate. The above results show that the adjuvant activity of QH-B declines, in a dose dependent manner with increasing degree of modification.

Cholesterol binds strongly to Quillaja saponin (For review see Höglund et al., 1989; Bomford 1980). This property of saponin is essential for the formation of iscom-matrix or iscoms. Trace amounts of radiolabelled cholesterol were added to non-modified or modified QH-B and binding capacities were examined. All modified QH-B preparations bound to cholesterol and formed saponin-lipid complexes as demonstrated by measurement of radioactivity in fractions isolated by sucrose gradient centrifugation and visualisation by electron

microscopy. These observations suggest that periodate oxidation of sugar residues does not influence the cholesterol binding capacity of Quillaja saponin.

The toxicity of modified QH-B was studied *in vivo* and *in vitro*. In vitro, the toxicity of periodate modified QH-B was examined on WEHI cells using a calorimetric method. This method is based on the capacity of mitochondrial dehydrogenase to convert the yellow dye MTT (tetrazolium salt) to a purple formazan product. The degree of conversion is used as a measurement of the metabolic activity of living cells. Non-modified QH-B inhibited the enzyme activity by 50% at a concentration of 5 µg/ml. Ten µg/ml of 2.5 or 5 mM sodium periodate modified QH-B and approximately 80 µg/ml of 50 mM periodate modified QH-B were required for 50% inhibition of enzyme activity. The haemolytic activity of QH-B on chicken red blood cells decreased proportionally with increasing concentrations of periodate treatment in a similar pattern as that seen for *in vitro* toxicity.

Mice were inoculated s.c. with 100 µg of non-modified QH-B or periodate modified QH-B (2.5 to 50 mM). The toxicities of the periodate treated QH-B preparations were studied in mice by recording the number of deaths and the time which elapsed between injection and death. Significantly decreased toxicity was only found in groups of mice inoculated with 25 or 50 mM sodium periodate QH-B and no decreased toxicity was observed in mice receiving QH-B modified with 2.5, 5 or 10 mM periodate. The above results indicate that periodate oxidation of QH-B decreases its toxicity, *in vivo*, in a dose- dependent manner. This study shows that carbohydrates, specially xylose and galactose, may have active roles in adjuvant activity and toxic effects of Quillaja saponin.

Taken together, the results of this study illustrate two important points, the first being that both the toxic effects and adjuvant activity of Quillaja saponin can be altered by modification of carbohydrates and the second being that periodate oxidation does not influence its cholesterol binding capacity.

Activation of antigen presenting cells by Quillaja saponaria formulations (papers III, IV and V)

Iscoms induce IL-1 and IL-6 more efficiently than iscom-matrix or free Quillaja components in vitro

Interleukin-1 (IL-1), IL-6 and IL-12 are proinflammatory cytokines which are mainly produced by APC. They have potent biological activities both in general and on the immune system in particular. The capacity of adjuvants to stimulate antigen presenting cells (APC) to produce proinflammatory cytokines is important in order to enhance the induction of immune response. QH 7.0.3 iscoms containing influenza virus envelope proteins (Flu-iscoms) have a significantly greater capacity to stimulate PEC to produce IL-1 and IL-6 *in vitro* than iscom-matrix and free Quillaja components. This is particularly evident and at low concentrations at which it was shown that free Quillaja saponin or

iscom-matrix did not induce detectable levels of cytokines. The QH 7.0.3 iscom-matrix was more efficient than QH 7.0.3 free components in induction of soluble IL-1α. There was no significant difference in the peak levels of IL-6 production from PEC following stimulation with iscom-matrix or free components. However, iscom-matrix was more efficient than free components in induction of IL-6 production at low concentrations of Quillaja saponin.

The biological activity of IL-1 was determined by a bioassay based on the proliferation of D10.G4.1 (D10) cells. The highest biological activity of soluble IL-1 (sIL-1) was measured in the culture supernatants of PEC stimulated with flu-iscoms. In general, biological activities of sIL-1 correlated well with the results from the immunoassay of IL-1α production. This suggests that the sIL-1α induced by Quillaja saponin formulations was bioactive. The only exception was the result observed in culture supernatants from cells stimulated by free components. These induced a comparatively higher sIL-1 bioactivity compared with the results from the quantitative assay for IL-1α, suggesting that these supernatants contained other factors than IL-1α which stimulated proliferation e.g. IL-1β.

The influence of antigen on the capacity of iscom-matrix to induce IL-1 and IL-6

The addition of flu-micelles to iscom-matrix influenced the PEC only marginally, inducing them to produce increased amounts of IL-1 compared to iscom-matrix alone. However, the flu-micelle by themselves i.e. without the addition of adjuvant did not induce detectable levels of soluble IL-1, a result which is in accord with our previous results showing that flu-micelles induce only membrane bound IL-1 (mIL-1) (Villacres-Eriksson et al., 1993). In contrast, flu-micelle alone efficiently induced IL-6 production by PEC.

The capacity of Quillaja saponin formulations to induce IL-1 and IL-6 is not due to contamination with LPS

LPS, a very bioactive product from bacteria, is a common contaminant in various biological products. It is a potent inducer of certain cytokines encompassing IL-1 and IL-6. To exclude the possibility that contaminating LPS in the Quillaja saponin formulations was influencing cytokine production, we added polymyxin B sulphate to the culture system in which cytokine production was measured. Induction of IL-1 production by LPS was completely abrogated by the addition of polymyxin B (Duff et al., 1982). However, in our study we noted that addition of polymyxin B abrogated induction of both IL-1 and IL-6 production by LPS. The added polymyxin B had no effect on the capacity of Quillaja saponin formulations or flu-micelles to induce IL-1 and IL-6. The results clearly demonstrate that the capacity of Quillaja saponin formulations to induce proinflammatory cytokines is not due to LPS contamination.

Iscoms are potent inducers of IL-6 and IL-12 in vivo

The induction of IL-1, IL-6 and IL-12 were studied by measuring their concentration in sera of mice injected with the various Quillaja saponin formulations. In contrast to IL-1 which could only be detected *in vitro*, IL-6 and IL-12 were detectable in serum after intravenous injection (i.v.) of mice with flu-iscoms. IL-6 was also detected in the sera of mice injected i.v. with iscom-matrix alone, i.e. with no antigen, and that level was significantly higher than that measured in sera of mice injected with iscom-matrix plus flu-micelles. This suggests that flu-micelles have a downregulating effect on IL-6 production *in vivo*. In contrast to the *in vitro* effect, flu-micelles without adjuvant did not induce detectable serum levels of IL-6. This discrepancy may be due to the fact that different cell populations are responding in the *in vitro* and *in vivo* system. PEC are used in the *in vitro* system, while for example Kupffer´s cells are among the more accessible in the *in vivo* system and would be expected to constitute a large part of the *in vivo* response.

Interleukin-12 is a glycoprotein (p70) composed of two covalently linked subunits of approximately 40 kDa (p40) and 35 kDa (p35). The expression of both IL-12 subunits is necessary for generation of high levels of biological activity. In contrast to p40, the biologically active IL-12 i.e. p70, which is mainly produced by monocytes, while the expression of p35 mRNA is almost ubiquitous and has been demonstrated in almost all cells and cell lines examined. Generally, increased expression of p40 is associated with biological activity (Wolf et al., 1994). The capacity of flu-iscoms to induce IL-12 *in vivo* was studied in different haplotypes of mice i.e. Balb/c, C57BL/6 and CBA. In general, Balb/c responded to flu-iscoms with the highest level of p40 response in serum. The serum response was measured by a specific immunoassay and a bioassay for IL-12. The bioassay measured the production of IFNγ by naive splenocytes following *in vitro* stimulation. C57BL/6 mice produced a similar serum levels of p40 to that seen in Balb/c mice but the bioactivity was significantly lower which might be due to a surplus production of p40 as discussed for LPS below. The induction of IL-12 was also studied after i.v. injection of flu-iscoms, flu-micelles and iscom-matrix plus flu-micelles in Balb/c mice and compared with LPS. The peak response for p40 was detected 3 h post injection with flu-iscoms and LPS. The highest biologically active IL-12 response was detected in sera of mice injected with flu-iscoms. However, iscom-matrix plus flu-micelles and flu-micelles alone also induced bioactive IL-12, but at a significantly and considerably lower level than that induced by flu-iscoms. Although, the level of p40 induced by LPS was greater than that induced by flu-iscoms, the level of bioactive IL-12 induced by iscoms was significantly higher. This may indicate that LPS stimulates production of surplus p40 and this excess exerts inhibitory effects on the binding of IL-12 to its receptor (Gillessen, et al., 1995).

Taken together, the above results suggest that the incorporation of antigen and adjuvant in the same particle increases the capacity to stimulate APC to produce cytokines i.e. IL-1, IL-6 and IL-12. The induction of these cytokines most likely

reflects an important facet of the adjuvant effect *in vivo*, and it should be taken into account that APCs are important initial target cells for adjuvants.

QH-A is more efficient than QH-C in induction of IL-1 and IL-6 in vitro
To study the capacity of novel Quillaja saponins in iscom-matrix to stimulate APC, we prepared five different iscom-matrix formulations and analysed for their respective capacities to induce production of IL-1 and IL-6 in PEC. The five iscom-matrix formulations were; QH 7.0.3 iscom-matrix, QH 5.0.5 iscom-matrix, QH 3.0.7 iscom-matrix, QH-C iscom-matrix and spikoside iscom-matrix. The iscom-matrix of QH 7.0.3, containing 70% QH-A, 0% QH-B and 30% QH-C, was the most efficient formulation, followed by QH 5.0.5 iscom-matrix which closely resemble QH 7.0.3 iscom-matrix in composition. The capacity of QH-A vs QH-C to induce IL-6 in free forms or in iscom-matrix was also studied. The highest amount of IL-6 was detected in the supernatant of PEC stimulated with QH-A iscom-matrix, indicating the greater activity of QH-A iscom-matrix in this respect compared with free QH-A, QH-C iscom-matrix and free QH-C. However, the peak responses for IL-6 production induced by QH 7.0.3 free components and QH 7.0.3 iscom-matrix were similar. These results indicate that iscom-matrix with a higher ratio of QH-A/QH-C stimulates generally PEC more efficiently to produce IL-1 and IL-6. Although the efficiency of QH-A, when incorporated into iscom-matrix increased, and the efficiency of QH-C decreased when incorporated alone. The efficiency of the formulation as a whole (QH 7.0.3) was equal whether it was in free or iscom-matrix form.

QH 7.0.3 iscom-matrix is superior to spikoside iscom-matrix in induction of IL-6 in vivo
The capacity of QH 7.0.3 iscom-matrix and spikoside iscom-matrix respectively to induce IL-6 production was also studied *in vivo*. The serum levels of IL-6 in mice 3 h post i.v. administration measured by ELISA indicate that the iscom-matrix with 7.0.3 has a greater capacity to induce IL-6 than spikoside iscom-matrix.

Cytokine neutralising mAbs affect antibody responses to flu-iscoms, flu-micelles and EBV gp 340 protein non-adjuvanted or adjuvanted with iscom-matrix
Specific cytokine-neutralising mAbs have been used *in vitro* and *in vivo* to study the specific effects of each cytokine on the immune system. In this study, we analysed the effects of neutralising anti-cytokine mAbs on the antibody response of mice following s.c. administration of flu-iscoms, flu-micelles without adjuvant, recombinant EBV gp340 protein plus iscom-matrix or EBV gp340 protein without adjuvant. Balb/c mice were injected s.c. with anti-IL-12 mAb, anti-IL-4 mAb or anti-IFN-γ mAb and one hour later these mice were injected with the experimental vaccine formulations. Treatment with the anti-IL-12 mAb C15.6 significantly reduced the total specific antibody response

induced by flu-iscoms. Neither the anti-IFN-γ mAb nor anti-IL-4 mAb modified the total specific antibody response induced by flu-iscoms. In general, the anti-IL-12 mAb showed a broader effect than both the anti-IFN-γ mAb and the anti-IL-4 mAb on antibody responses induced by flu-iscoms or EBV gp340 protein plus iscom-matrix. The anti-IL-12 mAb exerted its effect by reducing the levels of total antigen specific antibody as well as reducing the levels of IgG1, IgG2a, and IgG2b. The anti-IL-4 mAb influenced the response to iscoms in an unexpected manner, reducing the level of IgG2a and increasing the level of IgG1. In contrast, this mAb significantly decreased IgG1 production induced by flu-micelles or EBV gp340 protein with or without iscom-matrix as would be expected. The IgG1 response induced by flu-micelles or EBV gp340 protein was enhanced several fold by treatment with anti-IFNγ mAb again this was an expected result. Treatment of mice injected with EBV gp340 protein plus iscom-matrix with anti-IFNγ mAb had remarkable effects on the antibody responses. IgG2b production was significantly reduced and, as could be expected, total antibody and IgG1 responses were also reduced. The reduction in IgG2a response seen was, however, not expected. All neutralising anti-cytokine mAbs tested i.e. anti-IL-12 mAb C15.6, anti-IL-4 mAb and anti-IFN-γ mAb only had modifying effects on IgG1 responses induced by flu-micelles. From these results it could be concluded that the different cytokines affected IgG1 responses either by reduction or enhancement (for more detail see Table 1).

Taken together, the above results show that when an antigen is incorporated into iscoms or mixed with iscom-matrix the immune response is modified compared to that induced by the antigen alone i.e. without adjuvant. However, the intrinsic biological activities of the antigen in itself have potent effects on the outcome of the ensuing immune responses.

Table 1: The effects of cytokine-neutralizing antibody on the development of the primary antibody responses in Balb/c mice administered with different antigens and Quillaja adjuvant formulations.

	Anti-IL-4 mAb	Anti-INFg mAb	Anti-IL-12 mAb C15.6
Flu-iscoms	IgG2a(-) IgG1(+),	IgG2a and IgG3(-), IgG2b(+)	Total, IgG1 (-) IgG2a and IgG2b(-)
EBV gp340+ iscom-matrix	Total, IgG1 (-) IgG2a and IgG2b(-)	IgG1, IgG2a (-) and IgG2b(-)	Total, IgG1 (-) IgG2a and IgG2b(-)
Flu-micelles	IgG1(-)	IgG1(+)	IgG1 (+/-)
EBV gp340	Total, IgG1(-)	NE	IgG1(-)

Total=total antibody response
(-)=decrease in the antibody response
(+)=increase in the antibody response
(+/-)=weak positive
NE=no effect

Concluding Remarks

This thesis presents results of analyses on the immunological properties of Quillaja saponin and also introduces some methods by which these properties can be studied. The data presented in this thesis can be grouped order three headings:

1) Methods have been introduced for isolation and quantification of the three categories of components in iscoms having important roles in stimulation of the immune responses i.e. Quillaja saponin, lipids and antigens,

2) The effects of periodate modification of the carbohydrate moiety of the Quillaja saponin measured in terms of biological activities i.e. immunoenhancing capacity, toxicological activity and cholesterol binding capacity have been analysed.

3) The ability of Quillaja saponaria Molina formulations to stimulate APC to produce cytokines has been studied.

i. From the first study we conclude that the chloroform-methanol-water extraction method is a reproducible procedure suitable for isolation of Quillaja saponin and lipids in iscom-matrix or iscoms. With regard to the quantification of Quillaja saponin and lipids, the reverse phase HPLC gives reproducible and reliable results. Furthermore, reverse phase HPLC can also be used in order to quantify each component of the Quillaja saponin, resulting in the almost exact measurement of ratio of each component of importance for the induction of immune responses.

ii. Sodium periodate oxidation at acidic pH is a reproducible method for modification of the carbohydrates of Quillaja saponin in a dose dependent manner. Periodate treatment modifies Quillaja saponin by loss of some monosaccharides such as apiose, galactose and xylose. Immunoenhancing capacity and toxicity were shown to be reduced subsequent to this treatment. In general, it can be concluded that carbohydrates of Quillaja saponin play an active role in the adjuvant activity and toxicity of the saponin. However, the periodate modification of monosaccharides did not affect their cholesterol binding capacity.

iii. Quillaja saponin efficiently stimulate APC to produce proinflammatory cytokines i.e. IL-1, IL-6 and IL-12. These cytokines are crucial in the induction of a potent immune response. Iscoms are the most potent inducers of these cytokines compared with other Quillaja saponin formulations i.e. iscom-matrix and free Quillaja saponin. Therefore, it can be concluded that the presence of antigen and adjuvant in the same particle increases the capacity to stimulate APC to produce cytokines.

Various components of Quillaja saponins differ in their capacity to stimulate cytokine production by APC. For example, QH-A was more efficient in inducing IL-1 and IL-6 production than QH-C.
Of the iscom-matrix formulations, QH 7.0.3 composed of 70% QH-A, 0% QH-B and 30% QH-C was the most efficient in stimulating APC to produce cytokines. Additional unpublished data (Behboudi, S. manuscript in preparation) show that QH 7.0.3 iscom-matrix also has a greater capacity to induce antibody responses and T cell responses than other formulations including QH-A iscom-matrix, suggesting a synergistic effect between QH-A and QH-C at high of QH-A/QH-C ratio.
The effects of cytokine neutralising mAbs on the induction of antibodies by flu-iscoms or by EBV gp340 adjuvanted with iscom-matrix demonstrate that the Quillaja saponin exerts its immunoenhancing activity through both Th1 and Th2 types of responses and that IL-12 plays an important role in the immunoenhancing capacity of iscoms.

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